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# A TBC1D1$^{Ser231Ala}$ knockin mutation partially impairs 5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside- but not exercise-induced muscle glucose uptake in mice

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**Abbreviations:** AICAR, 5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside; AMPK, AMP-activated protein kinase; AS160, Akt substrate of 160 kDa (also known as TBC1D4); GAP, GTPase-activating protein; GLUT4, glucose transporter 4; PKB, protein kinase B (also known as Akt); TBC1D1, tre-2/USP6, BUB2, cdc16 domain family member 1.

**ABSTRACT**

**Hypothesis:** TBC1D1 is a Rab GTPase activating protein (RabGAP) that has been implicated in regulating GLUT4 trafficking. TBC1D1 can be phosphorylated by the AMP-activated protein kinase (AMPK) on $Ser^{231}$, which consequently interacts with 14-3-3 proteins. Given the key role for AMPK in regulating insulin-independent muscle glucose uptake, we hypothesized that TBC1D1 $Ser^{231}$ phosphorylation and/or 14-3-3 binding may mediate AMPK-governed glucose homeostasis.

**Methods:** Whole-body glucose homeostasis and muscle glucose uptake were assayed in mice bearing a TBC1D1$^{Ser231Ala}$ knockin mutation or harboring skeletal muscle specific AMPKα1/α2 double knockout mutations in response to an AMPK-activating agent, 5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside (AICAR). Exercise-induced muscle glucose uptake and exercise capacity were also determined in the TBC1D1$^{Ser231Ala}$ knockin mice.

**Results:** Skeletal muscle specific deletion of AMPKα1/α2 in mice prevented AICAR-induced hypoglycemia and muscle glucose uptake. The TBC1D1$^{Ser231Ala}$ knockin mutation also attenuated the glucose-lowering effect of AICAR in mice. Glucose uptake and cell surface GLUT4 content were significantly lower in muscle isolated from the TBC1D1$^{Ser231Ala}$ knockin mice upon stimulation with a submaximal dose of AICAR. However, this TBC1D1$^{Ser231Ala}$ knockin mutation neither impaired exercise-induced muscle glucose uptake nor affected exercise capacity in mice.

**Conclusions:** TBC1D1 $Ser^{231}$ phosphorylation and/or 14-3-3 binding partially mediates AMPK-governed glucose homeostasis and muscle glucose uptake in a context-dependent manner.

## INTRODUCTION

Type 2 diabetes has become pandemic in the last few decades, which urges novel therapeutics to prevent, treat or cure the disease. Since insulin sensitivity is generally decreased in type 2 diabetic patients, insulin-independent treatment is commonly considered to combat type 2 diabetes. The energy sensor AMP-activated protein kinase (AMPK) has been proposed as an attractive drug target for treatment of type 2 diabetes as its activation brings about many of the acute and chronic beneficial effects of exercise and promotes muscle glucose uptake in an insulin-independent mechanism [1, 2].

The heterotrimeric AMPK holoenzyme consists of a catalytic subunit α (α1 and α2), a regulatory subunit γ (γ1, γ2 and γ3), and a third subunit β (β1 and β2) that bridges α and γ subunits [1]. AMPK regulates whole-body glucose homeostasis by various mechanisms [1, 3, 4], one of which is via control of muscle glucose uptake by promoting translocation of the glucose transporter 4 (GLUT4) from its intracellular storage sites onto plasma membrane [5]. Whole-body deletion of AMPK α2 or β2 subunit, or overexpression of a dominant inhibitory mutant of AMPK in muscle, attenuated the hypoglycemic effect of AICAR in mice. Furthermore, genetic inactivation of AMPK α2, β2 or γ3 subunit, or overexpression of the dominant inhibitory mutant of AMPK, all inhibit AICAR-stimulated muscle glucose uptake [6-10]. However, the regulatory mechanism of AMPK-dependent GLUT4 translocation remains to be elucidated. GLUT4 also mediates insulin-stimulated glucose uptake into skeletal muscle and adipose, whose translocation in response to insulin is regulated by the protein kinase B (PKB) pathway [11].

Two related RabGAPs, AS160 (Akt substrate of 160 kDa) and TBC1D1, have been linked with type 2 diabetes and obesity, respectively [12-15]. These enzymes have also been implicated in regulating GLUT4 trafficking (reviewed by [16]). Both AS160 and TBC1D1 can be phosphorylated on multiple sites by protein kinases including PKB and AMPK (reviewed by [17]). Overexpression of an AS160-4P mutant (in which $Ser^{325}$, $Ser^{595}$, $Thr^{649}$ and $Ser^{758}$ were replaced by alanine) or a TBC1D1-3P mutant (in which alanine replaced $Ser^{489}$, $Thr^{499}$/$Ser^{501}$, and $Thr^{590}$) exerts a robust inhibition on insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes [18, 19]. In contrast, overexpression of a TBC1D1-4P mutant (in which alanine replaced $Ser^{231}$, $Thr^{499}$, $Thr^{590}$, and $Ser^{621}$) decreased contraction-induced glucose uptake in mouse skeletal muscle [20]. Upon phosphorylation, both AS160 and TBC1D1 bind to regulatory 14-3-3 proteins (reviewed by [17]). The AS160−14-3-3 interaction is regulated by insulin and

mainly mediated by phosphorylated Thr$^{649}$ on AS160 [21, 22], whereas 14-3-3 binding to TBC1D1 is dependent on AMPK and mainly requires phosphorylated Ser$^{231}$ on TBC1D1 [23, 24]. Moreover, 14-3-3 binding to AS160 has been implicated in participating insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes [21]. On the basis of these findings, we put forward a hypothesis that AS160−14-3-3 interaction is required for insulin-stimulated GLUT4 trafficking and glucose homeostasis while 14-3-3 binding to TBC1D1 mediates AMPK-dependent GLUT4 translocation and glucose homeostasis [17]. Towards addressing this hypothesis, we previously generated an AS160$^{Thr649Ala}$ knockin mouse model in which insulin-induced AS160-Thr$^{649}$ phosphorylation and its binding to 14-3-3s was prevented, and showed that the AS160$^{Thr649Ala}$ knockin mice exhibited insulin resistance mainly due to impaired GLUT4 translocation and glucose uptake into skeletal muscle in response to insulin [25]. In contrast, the AICAR-stimulated muscle glucose uptake and hypoglycaemia, which are independent of insulin, were normal in these mice [26].

In this study we utilized a recently-reported TBC1D1$^{Ser231Ala}$ knockin mouse model in which the knockin mutation prevents AMPK-dependent phosphorylation of TBC1D1-Ser$^{231}$ [27] to further test the above hypothesis.

## Materials and Methods

### Materials

Recombinant human insulin was bought from Novo Nordisk (Denmark) and AICAR from Toronto Research Chemicals (Canada). Protein G-Sepharose was from GE-Healthcare (UK). 2-deoxy-D-[1,2-$^{3}$H(N)]glucose and D-[1-$^{14}$C]-mannitol were from PerkinElmer (USA). All other chemicals were from Sigma-Aldrich or Sangon Biotech (China). Total TBC1D1 antibody is as previously described [23]. The commercial antibodies are listed in Supplementary Table 1.

### Mouse breeding and genotyping

The Ethics Committees, initially at University of Dundee and latterly at Nanjing University, approved all animal protocols. Mice were kept under a light/dark cycle of 12 h. The TBC1D1$^{Ser231Ala}$ knockin mice were as previously described [27]. The AMPKα1$^{f/f}$ and AMPKα2$^{f/f}$ mice were as previously described [28], and they were mated with each other to obtain AMPKα1/α2$^{f/f}$ mice. The AMPKα1/α2$^{f/f}$ mice were then mated with the Myf5-Cre mice [29] to obtain the AMPKα1/α2$^{f/f}$–Myf5-Cre mice that are the skeletal muscle-specific

AMPKα1/α2 knockout (AMPKα1/α2-mKO) mice. Genotyping was carried out using primers listed in Supplementary Table 2.

**Tissue lysis, immunoprecipitation, immunoblotting and 14-3-3 overlay**

Homoginization of mouse tissues and protein concentration measurement were carried out as previously described [26].

Immunoprecipitation of TBC1D1 proteins, immunoblotting and 14-3-3 overlay were carried out as previously described [23].

**AICAR tolerance test and insulin tolerance test**

Mice were partially fasted for 4 h prior to AICAR or insulin tolerance test. Afterwards, basal blood glucose was measured using a Breeze 2 glucometer (Bayer) via tail bleeding. Mice were intraperitoneally injected either with AICAR (0.25 mg/g) for AICAR tolerance tests, or with insulin (0.75 mU/g) for insulin tolerance tests. After injection of AICAR or insulin, blood glucose levels were measured at the indicated time intervals.

**Muscle incubation, glucose uptake and photolabelling of cell surface GLUT4 in isolated muscle**

Muscle isolation and glucose uptake were carried out as previously described [25]. Briefly, isolated muscles were incubated in KRB buffer (+/− AICAR) for 50 min. For biochemistry studies, muscles were then blotted dry and snap-frozen in liquid nitrogen for subsequent analysis. For glucose uptake, muscles were further incubated in KRB buffer (+/− AICAR) containing 2-deoxy-D-[1,2-$^{3}$H(N)]glucose and D-[1-$^{14}$C]-mannitol for 10 min. Radioisotopes in muscle lysates were determined using a Tri-Carb 2800TR scintillation counter (PerkinElmer). For the photolabelling experiment, cell surface GLUT4 in isolated EDL muscle was chemically tagged with the photolabel Bio-LC-ATB-BGPA and quantified as previously described [25].

**Treadmill exercise**

Two types of treadmill running tests, namely power test (short high-intensity run) and endurance test (long low-intensity run), were performed to assess maximal exercise performance in mice using a treadmill (Techman Soft, China) as previously described [30]. Briefly, mice were acclimated to treadmill running (5 min per day at 15 cm/sec, +5° slope and 0.3 mA electrical stimulation) for 5 days before the tests. For power tests, the treadmill was set with a +5° slope and 0.3 mA aversive electrical stimulation. Belt speed was set at 15 cm/sec initially and

increased by 2 cm/sec every minute. As for endurance tests, the treadmill was set with a +5° slope and 0.3 mA aversive electrical stimulation. Belt speed was set at 15 cm/sec initially and increased by 3 cm/sec every 12-minutes. Running tests were terminated when mice failed to re-engage on the treadmill despite aversive stimulation for more than 15 sec.

**Exercise-stimulated muscle glucose uptake *in vivo***

Exercise-stimulated muscle glucose uptake was carried out as previously described [31]. Briefly, mice were intraperitoneally injected with 10 μCi of 2-deoxy-D-[1,2-$^{3}$H(N)]glucose immediately prior to rest or exercise. For the exercise group, mice were subjected to 35 min of treadmill running (30 cm/sec belt speed, +10° slope). For the resting group, mice were kept in home cages for 35 min. After exercise or rest, mice were terminated and gastrocnemius and quadriceps muscles were harvested for further analysis. An assumption was made that systemic delivery of the tracer was similar in all animals within each experimental group since the appearance of 2-deoxy-D-[1,2-$^{3}$H(N)]glucose in the blood was not determined due to technical reasons. Muscle accumulation of phosphorylated 2-deoxy-D-[1,2-$^{3}$H(N)]glucose was determined and used for calculation of *in vivo* muscle glucose uptake rates as previously described [25].

**Histology and imaging**

Isolated soleus and EDL muscles were embedded in Tissue Freezing Medium (#14020108926, Leica), frozen in liquid nitrogen, and sectioned using a Leica RM2016 microtome. Muscle fiber type (I, IIa, and IIb/IIx) in soelus and EDL muscle was determined using myosin ATPase staining as previously described [32]. After staining, pictures were taken on sections using an Olympus BX53F microscope.

**Immunofluorescence staining and imaging**

Immunofluorescence staining of GLUT4 were performed in single muscle fibers as previously described [27]. Images were photographed using a Leica confocal microscope.

**Statistical analysis**

Data are given as mean ± SEM. Two-group comparisons were carried out via *t*-test, and multiple-group comparisons were performed with 2-way ANOVA using Prism software (GraphPad, San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$ that was indicated with an asterisk. NS indicates not significant.

**Results**

**Deletion of AMPKα1/α2 in skeletal muscle blunted the hypoglycemic effect of AICAR and AICAR-stimulated muscle glucose uptake**

It has been shown that overexpression of the dominant inhibitory mutant of AMPK in skeletal and cardiac muscle attenuated AICAR-induced hypoglycemia [9]. To further determine the contribution of skeletal muscle AMPK to AICAR-induced hypoglycemia, we generated an AMPKα1/α2-mKO mouse model in which both AMPK α1 and α2 subunits were specifically deleted in skeletal muscle. Deletion of AMPK α1 and α2 subunits caused a substantial decrease of TBC1D1-Ser$^{231}$ phosphorylation but did not alter expression of TBC1D1 and AS160 in skeletal muscle (Fig. 1a). Expression and phosphorylation of AMPKα and TBC1D1 remained normal in other tissues analyzed (Fig. 1b-c). Intraperitoneal injection of AICAR caused hypoglycemia in the AMPKα1/α2$^{f/f}$ control mice (Fig. 1d). Interestingly, this hypoglycemic effect of AICAR was blunted in the AMPKα1/α2-mKO mice (Fig. 1d). As expected, glucose uptake was completely inhibited in the AMPKα1/α2-deficient soleus and EDL muscle in response to AICAR (2 mmol/l) (Fig. 1e-f). Concomitantly, AICAR-stimulated GLUT4 translocation was impaired in EDL muscle from the AMPKα1/α2-mKO mice (Fig. 1g-h). These data confirm previous reports that AMPK controls AICAR-stimulated muscle glucose uptake and GLUT4 translocation [6-10, 33], and also demonstrate that muscle AMPK makes a large, if not complete, contribution to AICAR-induced hypoglycemia under the conditions used in this study.

**The TBC1D1$^{Ser231Ala}$ knockin mutation attenuated the hypoglycemic effect of AICAR**

Next, we employed the TBC1D1$^{Ser231Ala}$ knockin mice to investigate whether TBC1D1 mediates the AICAR-induced hypoglycemic effect at downstream of AMPK. Intraperitoneal injection of AICAR induced phosphorylation of AMPK and a well-established *bone fide* AMPK substrate ACC in knockin muscle to similar extents as in wild-type muscle (Fig. 2a). In contrast and as anticipated, AICAR-stimulated TBC1D1-Ser$^{231}$ phosphorylation was only detected in muscle extracts from the wild-type mice but not knockins (Fig. 2a). The AICAR-stimulated 14-3-3−TBC1D1 interaction assessed by a 14-3-3 overlay assay was also pronouncedly reduced (by over 80%) in TBC1D1 immunoprecipitates from the knockin muscle extracts as compared to the wild-type controls (Fig. 2a). As previously reported [27], expression of AS160 remained normal in TBC1D1$^{Ser231Ala}$ knockin muscle (Fig. 2a). Interestingly, the hypoglycemic effect of AICAR was significantly attenuated in both male and female TBC1D1$^{Ser231Ala}$ knockin mice (6 to 8-

week-old) (Fig. 2b-c). We previously showed that the TBC1D1$^{Ser231Ala}$ knockin mice were tolerant to glucose administration at a young age (less than 4-month-old) [27]. In agreement with this report, insulin injection lowered blood glucose levels to similar extents in the young TBC1D1$^{Ser231Ala}$ knockin mice and wild-type littermates (10 to 12-week-old) (Fig. 2d).

**AICAR-stimulated glucose uptake was decreased in skeletal muscle from the TBC1D1$^{Ser231Ala}$ knockin mice**

We first examined whether soleus and EDL muscles exhibit different sensitivity to AICAR in the glucose uptake assay since they have different fiber composition. To this end, two concentrations of AICAR were used to stimulate glucose uptake in wild-type soleus and EDL muscles. Glucose uptake rates stimulated by a low concentration of AICAR (0.25 mmol/l) were ~55% and ~94% of those stimulated by the high concentration of AICAR (2 mmol/l) in soleus and EDL muscles, respectively (Fig. 3a), suggesting that EDL muscle is more sensitive to AICAR treatment.

We next investigated whether the TBC1D1$^{Ser231Ala}$ knockin mutation impaired AICAR-stimulated muscle glucose uptake. Glucose uptake was normal in soleus and EDL muscles from the TBC1D1$^{Ser231Ala}$ knockin mice under basal conditions. Upon stimulation with AICAR (2 mmol/l), glucose uptake rates in the knockin soleus muscle were nearly 20% lower ($p$ = 0.0501) than those in the wild-type muscle (Fig. 3b). Upon stimulation with the high concentration of AICAR (2 mmol/l), glucose uptake rates were only marginally lower (not statistically significant) in the knockin EDL muscle than those in the wild-type muscle (Fig. 3c). Interestingly, upon stimulation with a low concentration of AICAR (0.15 mmol/l), glucose uptake rates in the knockin EDL muscle were significantly lower by ~17% than those in the wild-type muscle (Fig. 3c). These data show that the TBC1D1$^{Ser231Ala}$ knockin mutation moderately impaired AICAR-stimulated muscle glucose uptake.

Fiber composition of both soleus and EDL muscles from the TBC1D1$^{Ser231Ala}$ knockin mice had no obvious differences compared with the corresponding muscles from the wild-type littermates (Fig. 4a-c), indicating that muscle fiber type switch is not the cause of impaired AICAR-stimulated glucose uptake in skeletal muscle from the TBC1D1$^{Ser231Ala}$ knockin mice.

**AICAR-stimulated GLUT4 translocation was impaired in EDL muscle from the TBC1D1$^{Ser231Ala}$ knockin mice**

GLUT4 is the major glucose transporter that mediates AICAR-stimulated glucose uptake and it undergoes subcellular translocation onto the plasma membrane upon AICAR stimulation [9]. As previously reported [27], GLUT4 expression was normal in EDL muscle from the TBC1D1$^{Ser231Ala}$ knockin mice (Fig. 5a-b). However, GLUT4 contents on cell surface determined using a photolabeling method was only increased by ~25% in *ex vivo* EDL muscle from the TBC1D1$^{Ser231Ala}$ knockin mice upon stimulation with 0.15 mmol/l AICAR in contrast to an increase of ~53% in wild-type control muscle (Fig. 5c), suggesting that defects in GLUT4 translocation underlie the impaired glucose transport in skeletal muscle from the TBC1D1$^{Ser231Ala}$ knockin mice. We also confirmed that AICAR increased phosphorylation of AMPK and ACC in *ex vivo* EDL muscle from both genotypes to similar extents (Fig. 5a). As expected, AICAR stimulated TBC1D1-Ser$^{231}$ phosphorylation in *ex vivo* EDL muscle from the wild-type mice but not from the TBC1D1$^{Ser231Ala}$ knockins (Fig. 5a).

**The TBC1D1$^{Ser231Ala}$ knockin mutation did not impair exercise performance and exercise-induced muscle glucose uptake in mice**

It has been well established that AMPK regulates exercise performance and exercise-induced muscle glucose uptake [34]. We then sought to find out whether TBC1D1-Ser$^{231}$ phosphorylation might mediate these effects of AMPK. The TBC1D1$^{Ser231Ala}$ knockin mice displayed normal exercise performance in both power and endurance running tests (Fig. 6a-d). Phosphorylation of AMPK and ACC was increased in the exercised muscle from both genotypes to similar extents (Fig. 6e). In contrast, phosphorylation of TBC1D1-Ser$^{231}$ was only increased in the exercised muscle from wild-type mice but not from TBC1D1$^{Ser231Ala}$ knockins (Fig. 6e). Exercise robustly increased muscle glucose uptake, which was comparable in skeletal muscle from the TBC1D1$^{Ser231Ala}$ knockin mice and wild-type littermates (Fig. 6f-g). The unaltered exercise-induced muscle glucose uptake was consistent with the recently reported normal contraction-stimulated glucose uptake in skeletal muscle from the TBC1D1$^{Ser231Ala}$ knockin mice [27].

**Discussion**

Here we employed the TBC1D1$^{Ser231Ala}$ knockin mouse to study the *in vivo* function of TBC1D1-Ser$^{231}$ phosphorylation and its subsequent 14-3-3−binding in mediating AMPK-governed muscle glucose uptake. A major finding of this study is that TBC1D1-Ser$^{231}$

phosphorylation and/or its binding to 14-3-3s regulate AICAR-mediated glucose metabolism at both peripheral and whole-body levels downstream of AMPK.

AICAR is widely used as a tool compound to study AMPK signaling, and it lowers blood glucose in an AMPK-dependent manner when administered to mice. Overexpression of a kinase-dead mutant form of AMPKα2, or genetic ablation of AMPK α2, or β2 can all attenuate the hypoglycemic effect of AICAR [6-9]. The contribution of AMPK to AICAR-induced hypoglycemia in various organs may depend on fasting status of mice. For instance, our data demonstrate that muscle AMPK makes a large, if not complete, contribution to AICAR-induced hypoglycemia in mice subjected to partial fasting for 4 h. This hypoglycemic effect is most likely due to AICAR-stimulated glucose uptake in skeletal muscle, which is blunted in the AMPKα1/α2-mKO mice. However, in overnight-fasted mice, liver AMPK also partially mediates AICAR-induced hypoglycemia [35]. Our TBC1D1$^{Ser231Ala}$ knockin mouse is a whole-body knockin model, which has more lean mass and normal fat mass at a young age (less than 4-month-old) [27]. Even though skeletal muscle plays a dominant role in AICAR-induced hypoglycemia in mice subjected to partial fasting (4 h), it is still possible that the TBC1D1$^{Ser231Ala}$ knockin mutation might impair AICAR-induced hypoglycemia through multiple organs including skeletal muscle. The TBC1D1$^{Ser231Ala}$ knockin mice have higher plasma IGF1 levels that activate the PKB pathway in skeletal muscle. Consequently, the PKB activates the downstream mTOR pathway, but does not phosphorylate AS160 that is a key regulator for muscle glucose uptake [27]. We currently do not know whether IGF1 levels can affect AICAR-induced hypoglycemia in mice.

It is worthy of note that the AMPKα1/α2-mKO mice were completely resistant to the hypoglycemic effect of AICAR, and glucose uptake was blunted in isolated skeletal muscle from our AMPKα1/α2-mKO mice (this study) and other AMPK mouse models even with the high concentration of AICAR (2 mmol/l) [6-10]. In contrast, the TBC1D1$^{Ser231Ala}$ knockin mice were only partially resistant to the hypoglycemic effect of AICAR, and glucose uptake was impaired in their EDL muscle only in response to the low concentration of AICAR (0.15 mmol/l) but not the high concentration of AICAR (2 mmol/l). Moreover, the TBC1D1$^{Ser231Ala}$ knockin mutation did not impair contraction/exercise-stimulated glucose uptake in skeletal muscle (this study and [27]), which is partially mediated by AMPK [9, 34]. One possible explanation is that other potential AMPK sites such as Thr$^{499}$, Ser$^{621}$, Ser$^{660}$ and Ser$^{700}$ on TBC1D1 [19, 36] together with

Ser$^{231}$ might account for the regulation of glucose uptake in response to the high concentration of AICAR (2 mmol/l) or muscle contraction. In support of this notion, overexpression of a TBC1D1-4P mutant (in which Ser$^{231}$, Thr$^{499}$, Thr$^{590}$, and Ser$^{621}$ were mutated to Ala) decreased contraction-stimulated glucose uptake in mouse skeletal muscle [20]. A second possibility is that the related RabGAP AS160 might contribute to AICAR- or contraction-stimulated glucose uptake. AICAR as well as muscle contraction could stimulate Ser$^{595}$ phosphorylation of AS160 though its role in mediating AICAR- or contraction-stimulated glucose uptake remains to be established [22]. Third is the likelihood that factors other than TBC1D1 and AS160 might be responsible for muscle glucose uptake in response to the high concentration of AICAR (2 mmol/l) and to muscle contraction. For example, guanine nucleotide exchange factors (GEFs) antagonize effects of RabGAPs on activation of downstream Rabs, and can potentially regulate GLUT4 trafficking and glucose uptake. One such GEF, Dennd4C, has recently been identified as an important factor in regulating GLUT4 trafficking in adipocytes [37]. It is conceivable that such GEFs also exist in muscle and regulate GLUT4 translocation in response to AICAR or muscle contraction. Since the TBC1D1 Ser$^{231}$ phosphorylation and/or its binding to 14-3-3s only play a moderate role in mediating AMPK-governed muscle glucose uptake, these knockin mice should be useful in helping to identify other putative mediators of AMPK-regulated muscle glucose uptake in the future.

A growing body of evidence shows that genetic manipulation or mutation of the related RabGAPs, TBC1D1 and AS160, regulate GLUT4 by modulating both its expression and translocation in a complex manner. Insulin-stimulated GLUT4 translocation onto plasma membrane was impaired in the AS160$^{Thr649Ala}$ knockin mice although expression of the transporter was elevated [25]. In contrast, expression of GLUT4 was decreased in various muscles and adipose tissue of the AS160 deficient mouse models [38-40] as well as in skeletal muscles from human patients who harbor a pre-mature stop codon Arg$^{684}$Ter on AS160 [15]. Similarly, here we show that the TBC1D1$^{Ser231Ala}$ knockin mutation did not affect GLUT4 expression but attenuated GLUT4 translocation onto plasma membrane in response to AICAR, whereas a natural TBC1D1 deficient mutant mouse and various TBC1D1 knockout mice had reduced levels of GLUT4 in their skeletal muscles [14, 41-44]. Interestingly, the reduced GLUT4 levels in one of the TBC1D1 knockout mouse models caused a decrease in exercise-stimulated glucose uptake in nonoxidative muscle fibers and consequently impaired exercise

endurance [44]. It has been recently shown that the loss of AS160 GAP activity accelerates lysosomal degradation of GLUT4 [40]. A key question remaining to be answered in all the TBC1D1 mouse models is whether the effects on GLUT4 translocation or expression are dependent on the GAP activity of TBC1D1.

In summary, the TBC1D1$^{Ser231Ala}$ knockin mutation impacts on insulin-independent/AICAR-mediated whole-body glucose homeostasis in mouse at least in part through impairing muscle GLUT4 translocation and glucose uptake.

**Author contributions**

Q.L.C., B.X.X., S.S.Z., P.R., Y.S., S.D., L.C., C.Q., and M.L. performed experiments, analyzed data, reviewed and edited the manuscript. K.S. and C.M. were involved in the early stages of this study, and reviewed and edited the manuscript. S.C. and H.Y.W. designed and performed experiments, analyzed data, and wrote the manuscript.

**Conflict of interest**

K.S. is an employee of the Nestlé Institute of Health Sciences S.A., Switzerland.

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**Figure legends**

**Figure 1 AICAR-stimulated glucose clearance and muscle glucose uptake in AMPKα1/α2-mKO mice**

a-c. Expression and phosphorylation of AMPKα, TBC1D1 and AS160 in skeletal muscle (a), heart (b) and liver (c).

d. AICAR tolerance test of male AMPKα1/α2$^{f/f}$ and AMPKα1/α2-mKO mice (6-8-week-old). n = 5-6.

e. Glucose uptake in soleus muscle *ex vivo* in response to AICAR (2 mmol/l). † indicates $p < 0.05$ (AMPKα1/α2$^{f/f}$ AICAR vs AMPKα1/α2$^{f/f}$ basal). n = 7.

f. Glucose uptake in EDL muscle *ex vivo* in response to AICAR (2 mmol/l). † indicates $p < 0.05$ (AMPKα1/α2$^{f/f}$ AICAR vs AMPKα1/α2$^{f/f}$ basal). n = 5-6.

g-h. GLUT4 staining in EDL muscle fibers in response to AICAR (2 mmol/l). g, representative images. h, quantitative data of plasma membrane (PM) GLUT4. At least 100 bracketed regions (examples shown in g) from ~20 muscle fibers were quantified per condition/genotype. Bars indicate 2 μm in length. † indicates $p < 0.05$ (AMPKα1/α2$^{f/f}$ AICAR vs AMPKα1/α2$^{f/f}$ basal).

Statistical analyses were performed via t-test for d, or via two-way ANOVA for e, f and h.

**Figure 2 Glucose clearance in the TBC1D1$^{Ser231Ala}$ knockin mice after intraperitoneal injection with AICAR or insulin**

a. Ser$^{231}$ phosphorylation and 14-3-3 binding of TBC1D1 in skeletal muscle upon AICAR stimulation. TBC1D1 proteins were immunoprecipitated from lysates of TA muscle from mice intraperitoneally injected with either saline (basal) or AICAR (0.25 mg/g), and subject to immunoblotting analysis. Total and phosphorylated ACC, AMPK and AS160 were measured in muscle lysates with GAPDH as internal control.

b. AICAR tolerance test of male mice at 6-8 weeks of age. n = 9-12.

c. AICAR tolerance test of female mice at 6-8 weeks of age. n = 6.

d. Insulin tolerance test of male mice at 10-12 weeks of age. n = 6-7.

Statistical analyses were carried out via t-test.

**Figure 3 Glucose uptake in skeletal muscle *ex vivo* upon AICAR stimulation**

a. Glucose uptake in EDL and soleus muscle *ex vivo* in response to AICAR (0.25 mmol/l and 2 mmol/l). n = 4.

b. Glucose uptake in soleus muscle *ex vivo* in response to AICAR (2 mmol/l). n = 6-7. * indicates $p < 0.05$ (WT AICAR vs WT basal), and † indicates $p < 0.05$ (TBC1D1$^{Ser231Ala}$ AICAR vs TBC1D1$^{Ser231Ala}$ basal).

c. Glucose uptake in EDL muscle *ex vivo* in response to AICAR (0.15 mmol/l and 2 mmol/l). n = 15-21. † indicates $p < 0.05$ (WT AICAR vs WT basal), and ‡ indicates $p < 0.05$ (TBC1D1$^{Ser231Ala}$ AICAR vs TBC1D1$^{Ser231Ala}$ basal).

Statistical analyses were performed via t-test for a, or via two-way ANOVA for b and c.

**Figure 4 Muscle fiber composition in soleus and EDL muscles from the wild-type and TBC1D1$^{Ser231Ala}$ knockin mice**

a. Representative images of myosin ATPase staining in soleus and EDL muscle for determination of muscle fiber composition. Bars indicate 50 μm in length.

b-c. Percentage of different muscle fibers in soleus (b) and EDL (c) muscle. soleus, n = 4-5; EDL, n = 5-7. Statistical analyses were performed via t-test.

**Figure 5 Cell surface GLUT4 contents in EDL muscle *ex vivo* upon AICAR stimulation**

a. Expression and phosphorylation of TBC1D1, ACC and AMPK, and expression of GLUT4 in EDL muscle *ex vivo* upon AICAR stimulation. Phosphorylation of TBC1D1-Ser$^{231}$ was measured on immunoprecipitated TBC1D1 proteins.

b. Quantitation of GLUT4 proteins in EDL muscle. The GLUT4 blot from Fig. 5a was used for quantitation. n = 6.

c. Cell surface GLUT4 levels in EDL muscle *ex vivo* in response to AICAR (0.15 mmol/l). n = 4. Representative blots are shown.

Statistical analyses were performed via t-test for b, or via two-way ANOVA for c.

**Figure 6 Exercise performance and exercise-stimulated muscle glucose uptake *in vivo***

a-d. Treadmill running exercise in the wild-type and TBC1D1$^{Ser231Ala}$ mice (3-month-old). a (male) and b (female), power test. c (male) and d (female), endurance test. n = 6-15.

e. Total and phosphorylated TBC1D1, ACC and AMPK in gastrocnemius muscle from rested or treadmill-exercised mice (female, 7-8-week-old).

f-g. *In vivo* muscle glucose uptake in gastrocnemius (f) and quadriceps (g) from rested or treadmill-exercised wild-type and TBC1D1$^{Ser231Ala}$ mice (female, 7-8-week-old). Resting group, n = 3-5; Running group, n = 6-7. * indicates $p < 0.05$ (WT running vs WT resting), and † indicates $p < 0.05$ (TBC1D1$^{Ser231Ala}$ running vs TBC1D1$^{Ser231Ala}$ resting).

Statistical analyses were performed via t-test for a-d, or via two-way ANOVA for f-g.